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Metabolomic Signature of Angiopoietin-Like Protein 3 Deficiency in Fasting and Postprandial State

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Objective—Loss-of-function (LOF) variants in the *ANGPTL3* (angiopoietin-like protein 3) have been associated with low levels of plasma lipoproteins and decreased coronary artery disease risk. We aimed to determine detailed metabolic effects of genetically induced ANGPTL3 deficiency in fasting and postprandial state.

Approach and Results—We studied individuals carrying S17X LOF mutation in *ANGPTL3* (6 homozygous and 32 heterozygous carriers) and 38 noncarriers. Nuclear magnetic resonance metabolomics was used to quantify 225 circulating metabolic measures. We compared metabolic differences between LOF carriers and noncarriers in fasting state and after a high-fat meal. In fasting, ANGPTL3 deficiency was characterized by similar extent of reductions in LDL (low-density lipoprotein) cholesterol (0.74 SD units lower concentration per LOF allele [95% CI, 0.42–1.06]) as observed for many TRL (triglyceride-rich lipoprotein) measures, including VLDL (very-low-density lipoprotein) cholesterol (0.75 [95% CI, 0.45–1.05]). Within most lipoprotein subclasses, absolute levels of cholesterol were decreased more than triglycerides, resulting in the relative proportion of cholesterol being reduced within TRLs and their remnants. Further, β-hydroxybutyrate was elevated (0.55 [95% CI, 0.21–0.89]). Homozygous *ANGPTL3* LOF carriers showed essentially no postprandial increase in TRLs and fatty acids, without evidence for adverse compensatory metabolic effects.

Conclusions—In addition to overall triglyceride- and LDL cholesterol–lowering effects, ANGPTL3 deficiency results in reduction of cholesterol proportion within TRLs and their remnants. Further, *ANGPTL3* LOF carriers had elevated ketone body production, suggesting enhanced hepatic fatty acid β-oxidation. The detailed metabolic profile in human knockouts of *ANGPTL3* reinforces inactivation of *ANGPTL3* as a promising therapeutic target for decreasing cardiovascular risk.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2019;39:665-674. DOI: 10.1161/ATVBAHA.118.312021.)**

Key Words: biomarkers ■ fasting ■ humans ■ lipoproteins ■ metabolomics

Considerable risk for cardiovascular disease (CVD) persists in individuals treated with LDL (low-density lipoprotein) cholesterol–lowering statin and *PCSK9* inhibitor therapies.[1] Experimental models and human genetics have indicated that TRLs (triglyceride-rich lipoproteins) play a causal role in the pathogenesis of CVD.[2] Post hoc analyses of statin trials have further shown that reductions of TRL are associated with lower cardiovascular event risk independently of the LDL cholesterol reduction achieved from statins.[3,4] These observations have accelerated the development of novel TRL-lowering therapeutics for CVD prevention, with drug targets informed by recent discoveries from genetic studies.[5] These include identification of loss-of-function (LOF) mutations in the *ANGPTL3* (angiopoietin-like protein 3) causing familial combined hypolipidemia (FHBL2; OMIM No. 605019).[6,7] This Mendelian condition is characterized by simultaneous presentation of low circulating concentrations of triglycerides, LDL cholesterol, and HDL (high-density lipoprotein) cholesterol levels, as well as potential beneficial effects on glucose metabolism.[7]

ANGPTL3 is a protein secreted by the liver, and its deficiency was first identified in a hypolipidemic mouse strain.[8] The ANGPTL3 protein is as a potent inhibitor of lipoprotein lipase—a primary factor that clears TRL from the circulation.[9] Reduced ANGPTL3 also reduces hepatic apo (apolipoprotein) B secretion and increased hepatic LDL uptake, leading to reduced plasma LDL cholesterol levels.[10] ANGPTL3 further acts to inhibit endothelial lipase, which may contribute to the low HDL cholesterol levels in *ANGPTL3* LOF carriers.[11] The first human *ANGPTL3* LOF mutations described were nonsense mutations S17X and E129X in the first exon of the gene.[6] Since the initial

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Nonstandard Abbreviations and Acronyms	
ANGPTL3	angiopoietin-like protein 3
Apo	apolipoprotein
CVD	cardiovascular disease
GlycA	glycoprotein acetyl
HDL	high-density lipoprotein
iAUC	incremental area under the curve
IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
LOF	loss-of-function
NMR	nuclear magnetic resonance
TRL	triglyceride-rich lipoprotein
VLDL	very-low-density lipoprotein

publications on these mutations, several other *ANGPTL3* LOF mutations have been characterized.[12–14]

Individuals with complete ANGPTL3 deficiency have been reported to lack significant coronary atherosclerotic plaques.[15] Exome sequencing of large epidemiological cohorts has further shown that heterozygous carriers of *ANGPTL3* LOF variants have a 35% to 40% reduced risk of coronary artery disease compared with the general population.[15,16] Moreover, no increased prevalence in fatty liver disease, or other apparent adverse health effects, has been identified for *ANGPTL3* LOF carriers, including homozygote carriers with complete ANGPTL3 deficiency.[7,17] Thus, *ANGPTL3* is a promising therapeutic target for lowering CVD risk, and clinical trials of monoclonal antibodies or antisense oligonucleotides for *ANGPTL3* inhibition have recently shown to lead to substantial lowering of circulating triglycerides and LDL cholesterol levels.[16,18]

Despite the promising results from phase 1 trials targeting *ANGPTL3*,[16,18] a number of important questions of the molecular effects of *ANGPTL3* inhibition remain. First, it is unclear which specific lipid components of TRL are mostly affected by ANGPTL3 deficiency, and thus the underlying mechanism of reduced CVD risk is incompletely understood. Second, the effects of ANGPTL3 deficiency on many emerging biomarkers for cardiometabolic risk have not been addressed.[9] We have previously shown that nuclear magnetic resonance (NMR) metabolomics is a powerful method to characterize the fine-grained effects of *PCSK9* and *HMGCR* genetic variants on a lipid metabolism and other metabolic pathways, with a close match to the changes observed in statin trials.[19,20] Here, we use NMR metabolomics to characterize the systemic effects of ANGPTL3 deficiency on detailed measures of lipoprotein composition, fatty acids, and circulating metabolites. Because most people are in a nonfasting state for the majority of the day, studying the effects on postprandial metabolism may further contribute to explain the cardioprotective mechanism of ANGPTL3 deficiency. We have previously reported highly reduced postprandial response in triglycerides among *ANGPTL3* LOF homozygotes.[21] Here, we further examined the detailed metabolic effects of ANGPTL3 deficiency after a high-fat meal.

Materials and Methods

The data that support the findings of this study are available from M.A. (marcello.arca@uniroma1.it) on reasonable request.

Study Cohort of *ANGPTL3* LOF Carriers

An overview of the study design in shown in the Graphic Abstract. The study population and the design of the oral fat tolerance challenge have been described in detail elsewhere.[21] Briefly, 6 homozygous and 32 heterozygous carriers of *ANGPTL3* S17X LOF mutation (henceforth *ANGPTL3* LOF) and 38 noncarriers were considered in the present study. Clinical examinations and blood sample drawings were performed after an overnight fast, after which participants underwent an oral fat tolerance test. The test meal (muffin prepared with olive oil, eggs, ricotta cheese, nuts, cocoa, wheat flour, and skimmed milk) consisted of 73 g fat, 52 g carbohydrate, 22 g protein, and 145 mg cholesterol. Blood samples were drawn before the test meal and at 2, 4, and 6 hours after the meal. The Ethical Committee of Sapienza University of Rome approved the study protocol, and all study participants provided their informed consent.

Lipid and Metabolite Quantification

Frozen EDTA plasma samples from fasting state and those collected during the oral fat tolerance test were used for metabolomic analyses. Metabolic biomarkers were quantified using proton NMR metabolomics (Nightingale Health, Ltd, Helsinki, Finland). This method provides simultaneous quantification of routine lipids, lipoprotein subclass profiling with lipid concentrations within 14 subclasses, fatty acid composition, and various low-molecular metabolites, including amino acids, ketone bodies, and gluconeogenesis-related metabolites in molar concentration units. To provide indication of which peaks give origin to the quantified metabolite data, representative NMR spectra of study participants at fasting and postprandial state are shown in Figure I in the online-only Data Supplement. More detailed metabolite assignment across complete spectral regions used has been published previously.[19] Details of the experimentation and applications of the NMR metabolomics platform have been described previously.[19,20,22,23]

To compare measurements obtained by the Nightingale NMR assay and clinical chemistry, we calculated Pearson pairwise correlations between some biomarkers that were obtained with both methods in our dataset; LDL and HDL cholesterol, apoA1, and apoB. The correlations were high for all measures in all timepoints (0.93–0.95 for LDL cholesterol, 0.90–0.95 for HDL cholesterol, 0.79–0.84 for apoA1, and 0.80–0.81 for apoB). Holmes et al[24] have previously reported slightly lower estimates of correlation for LDL and HDL cholesterol (0.86 and 0.88, respectively) but higher estimates for apoA1 and apoB (0.87 and 0.90, respectively) based on nonfasting samples in a large biobank study.

Statistical Analysis

We first evaluated associations between *ANGPTL3* LOF carrier status and fasting metabolite measures. Before analyses, the concentrations of all metabolic markers were log-transformed and scaled to SD units to enable comparison of results for measures with different units and across wide ranges of concentrations. As the primary analysis, we examined an additive model for association between metabolites and the 3 genotype classes (*ANGPTL3* LOF homozygotes, heterozygotes, and noncarriers). For each metabolic measure, the concentration difference (in log-transformed and subsequently SD-scaled units) per *ANGPTL3* LOF allele was calculated using linear regression adjusted for age and sex. As a secondary analysis, we studied the associations of complete *ANGPTL3* knockouts and fasting metabolites using a recessive model (homozygous carriers versus heterozygotes and noncarriers combined). Because of the correlated nature of metabolomics measures, we used false discovery rate correction and defined false discovery rate–corrected $P<0.05$ as statistically significant association.

To evaluate postprandial responses in metabolites, we calculated means and SEs by *ANGPTL3* LOF carrier status at each timepoint. Postprandial dynamics in the 3 genotype classes were compared using net incremental area under the curve (iAUC) statistics (area under the curve that differs from the fasting value) with the trapezoidal rule.[25] The differences in iAUCs between the 3 genotype classes were tested with *t* tests.

Results

Study Participants

The clinical characteristics of the study participants are summarized in the Table. *ANGPTL3* LOF carriers and noncarriers were comparable for age and sex. Heterozygous *ANGPTL3* LOF carriers had higher body mass index and waist circumference than noncarriers; however, these metrics did not differ between homozygous and noncarrier subjects. Plasma levels of ANGPTL3 protein were undetectable in homozygotes, whereas heterozygotes had 51% lower concentration compared with that of noncarriers. Previous report has shown that the 3 genotype classes were comparable for dietary intake, physical activity, smoking prevalence, and use of anti-inflammatory medications.[21] However, we note that in the current study, the plasma of 1 homozygous individual ran out and 1 additional heterozygous individual was tested for the oral fat load after the conclusion of the previous study.[21] Descriptive statistics for all metabolic biomarkers tested are reported in Table I in the online-only Data Supplement.

Effects of *ANGPTL3* LOF on Lipids and Metabolites at Fasting

Compared with noncarriers, carriers of *ANGPTL3* LOF mutations had substantially reduced levels in almost all lipid measures assayed by NMR metabolomics. These include lower concentrations of routine lipid measures, as well as the cholesterol and triglyceride levels in major subfractions, and reduced concentrations of apoB and apoA1 (Figure 1; Table II in the online-only Data Supplement). Scaled to the same variation in each lipid measure (ie, in units of SD), we observed comparable extent of lowering effects on LDL cholesterol levels (−0.74 SD units of concentration per allele [95% CI, −1.06 to −0.42]) as observed for VLDL (very-low-density lipoprotein) cholesterol levels (−0.75; 95% CI, −1.05 to −0.45) and other measures of TRL. The lowering effect size on total plasma triglycerides was −0.62 (95% CI, −0.93 to −0.32). In absolute concentrations, these lowering effects correspond to 0.28 mmol/L lower for LDL cholesterol, 0.13 mmol/L lower for VLDL cholesterol, and 0.20 mmol/L for total triglycerides. Also, HDL cholesterol and other lipid measures in HDL particles were lowered to a similar extent when scaled to SD concentrations of same variation. The effects of *ANGPTL3* LOF on all tested metabolites are shown in Figures II and III in the online-only Data Supplement.

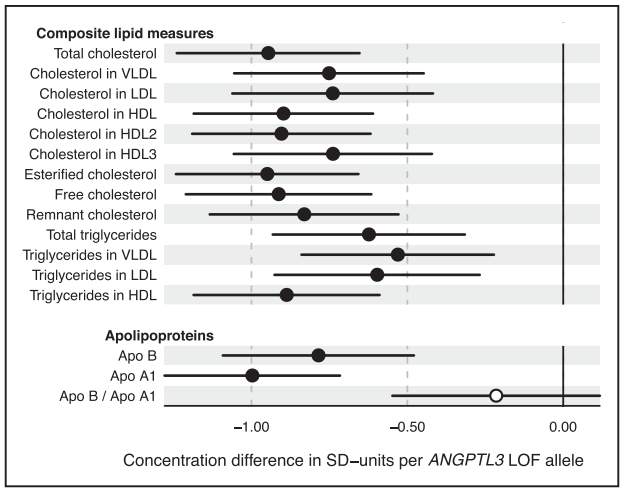

Figure 1. Effects of *ANGPTL3* (angiopoietin-like protein 3) loss-of-function (LOF) variant on fasting lipoprotein lipids and Apo (apolipoproteins). Effect estimates are shown as difference in SD-scaled concentration units per *ANGPTL3* S17X allele (additive model). Error bars indicate 95% CIs. Filled and open circles denote false discovery rate–corrected *P* value for association below and above 0.05, respectively. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.

Zooming in on the more specific effects of *ANGPTL3* LOF on lipoprotein subclass measures, we observed substantial reduction of cholesterol concentration within all lipoprotein subclasses (Figure 2A). The lowering effects were somewhat stronger for IDL (intermediate-density lipoprotein) in comparison with other apoB-100–containing particles. For HDL subclasses, cholesterol levels were most prominently lowered in medium-sized HDL particles. Scaled to the same variation, the extent of cholesterol lowering in all 10 apoB-containing subclasses was broadly similar to that observed for the routine lipid measures. Analogously, the triglyceride concentration in all lipoprotein subclasses (except for small HDL) was reduced; however, the triglyceride lowering was to a somewhat smaller extent for IDL and other similarly sized lipoprotein particles than that observed for cholesterol. Thus, even though the absolute triglyceride levels in lipoprotein particles were reduced, the relative proportion of triglycerides in TRL particles was actually increased because of ANGPTL3 deficiency. Concordantly, the cholesterol proportion of medium-sized and small VLDL and IDL particles decreased (Figure 2A, bottom). The proportional measures of lipid content in the lipoprotein subclasses are further illustrated in Figure 2B, which depicts the overall lipid composition for selected lipoprotein subclasses in *ANGPTL3* LOF carriers and noncarriers. For example, the proportion of cholesterol in small VLDL was 36% in noncarriers, 34% in *ANGPTL3* LOF heterozygotes, and 26% in homozygotes. The cholesterol proportion was also reduced in large LDL (67%, 66%, and 62%, respectively), whereas in large HDL, *ANGPTL3* LOF homozygotes had the highest proportion of cholesterol (55% versus 47% and 46% in *ANGPTL3* LOF

Table 1. Characteristics of Study Participants

	Noncarriers	*ANGPTL3* Heterozygous Carriers	*ANGPTL3* Homozygous Carriers
n	38	32	6
Age, y	47.0 (12.6)	50.8 (14.5)	53.5 (24.5)
Sex, females	21 (55.3)	15 (46.9)	4 (66.7)
Body mass index, kg/m^2	26.3 (4.2)	28.8 (4.4)*	27.8 (5.0)
Waist circumference, cm	92.5 (10.5)	100.7 (11.8)*	97.6 (15.0)
Statin use	1 (2.6)	2 (6.3)	0
Fasting ANGPTL3 protein concentration, ng/mL	208.7 (80.3)	108.3 (60.1)	0

Data are presented as mean (SD) or number of individuals (%). ANGPTL3 indicates angiopoietin-like protein 3.

*P <0.05 when compared with noncarriers.

heterozygotes and noncarriers, respectively). These values also demonstrate larger differences in lipoprotein measures between heterozygotes and homozygotes than between noncarriers and heterozygotes, suggesting more prominent effects of complete knockout of *ANGPTL3* for these lipoprotein subclasses compared with the effects expected from a linear dose-response relation.

The substantial lowering effects of ANGPTL3 deficiency on lipoprotein lipids may influence both absolute concentrations of circulating fatty acid and their relative proportions. We found that *ANGPTL3* LOF carriers displayed a significant reduction of total fatty acids, including saturated, monounsaturated, and polyunsaturated omega-3 and omega-6 fatty acids (Figure 3). Assessment of the proportions of fatty acid classes (ie, their ratios relative to total fatty acids) indicated an elevation of the proportion of saturated fatty acids and a reduction in the proportion of omega-3 fatty acids.

To assess potential nonlipid effects of ANGPTL3 deficiency, we examined the changes in circulating amino acids, glycolysis and gluconeogenesis substrates and products, ketone bodies, and other metabolites quantified simultaneously alongside lipid measures by the NMR metabolomics platform. Several of these nonlipid biomarkers also showed significant association with *ANGPTL3* LOF carrier status (Figure 3). The ketone body β-hydroxybutyrate—a biomarker of hepatic fatty acid β-oxidation—was markedly elevated in *ANGPTL3* LOF carriers (0.55 SD per allele; 95% CI, 0.21–0.89). Further, the

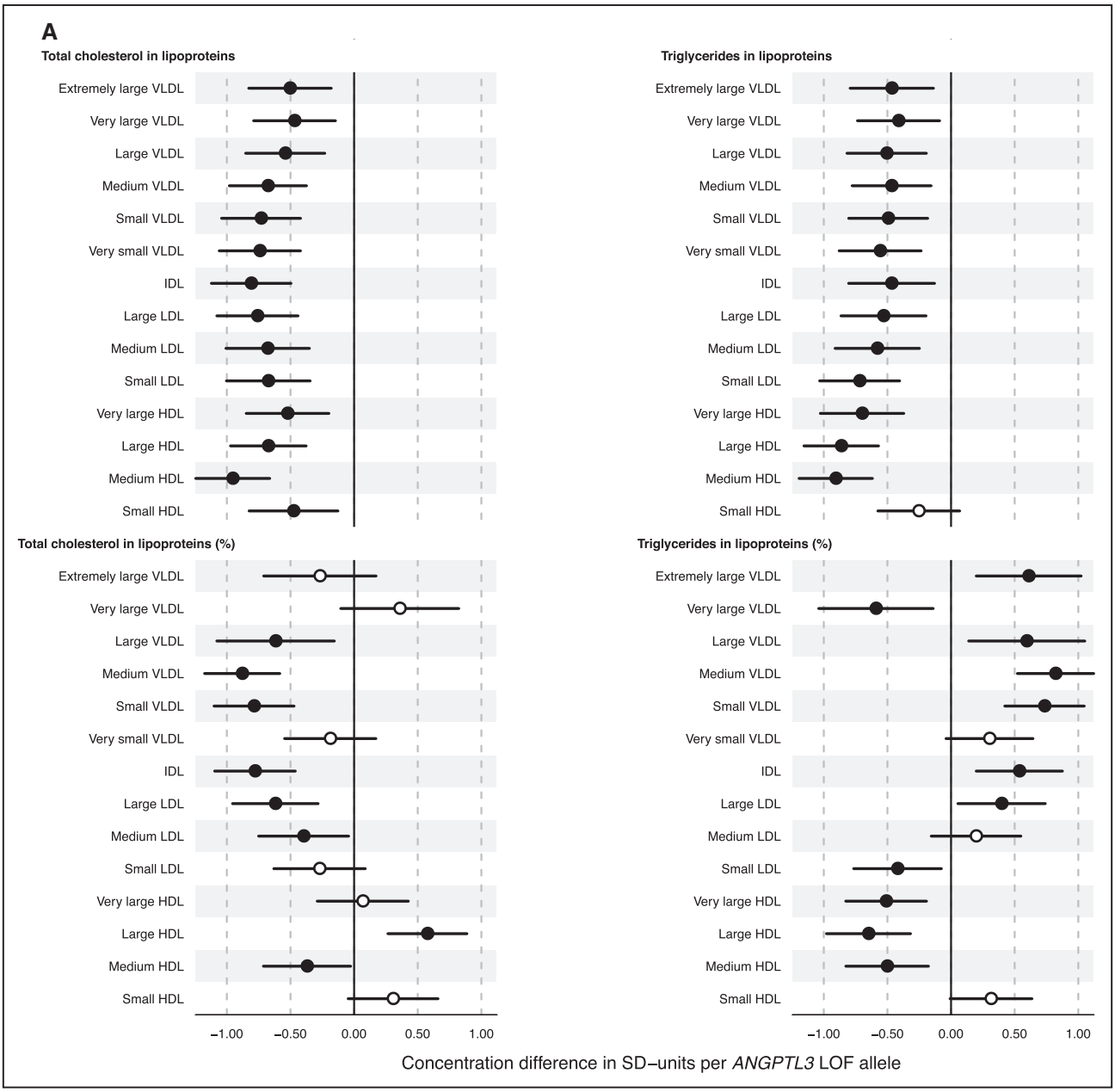

Figure 2. Effects of *ANGPTL3* (angiopoietin-like protein 3) loss-of-function (LOF) variant on fasting cholesterol and triglycerides concentrations in lipoprotein subclasses and their composition. **A**, Effect estimates are shown as difference in SD-scaled concentration units per *ANGPTL3* S17X allele (additive model). Error bars indicate 95% CIs. Filled and open circles denote false discovery rate–corrected *P* value for association below and above 0.05, respectively. (*Continued*)

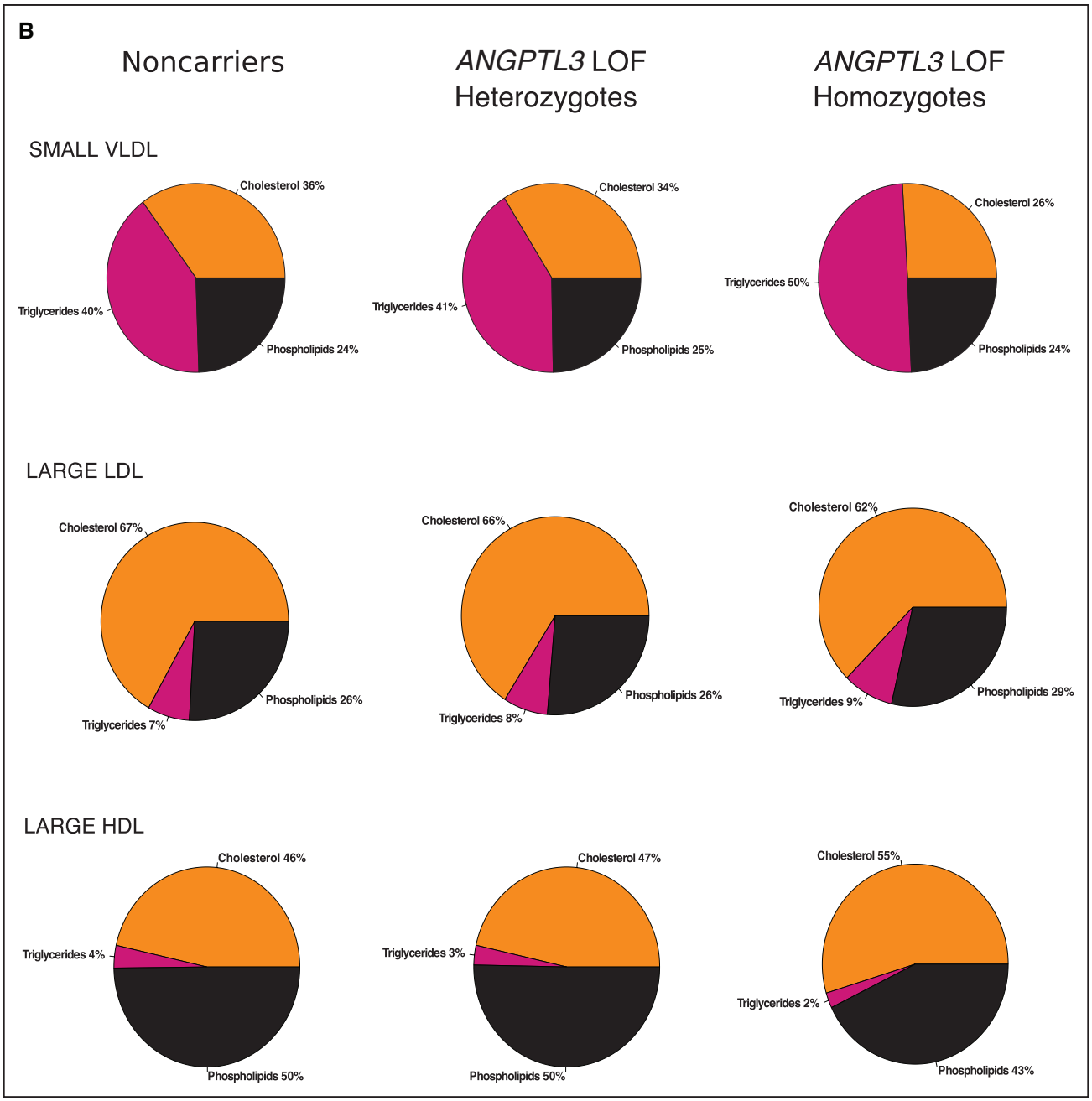

Figure 2 Continued. B, Average lipid content of selected lipoprotein subclasses in *ANGPTL3* LOF carriers and noncarriers are depicted to further illustrate the effects on lipoprotein composition. HDL indicates high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.

energy metabolism intermediate acetate was reduced (−0.56 SD per allele; 95% CI, −0.90 to −0.22) and citrate levels displayed a similar tendency. Branched-chained and aromatic amino acids, which are biomarkers for increased type 2 diabetes mellitus and CVD risk,[23,26] did not differ between *ANGPTL* LOF carriers and noncarriers. Further, we observed no differences in creatinine—an indicator of kidney function—or in GlycA (glycoprotein acetyl) level—a marker of chronic inflammation.

In analyses employing a recessive model, there was evidence for individuals with complete ANGPTL3 deficiency having more than twice the lipid-lowering effects of heterozygote *ANGPTL3* LOF carriers. For example, homozygous *ANGPTL3* LOF carriers had on the average −1.97 (95% CI, −2.69 to −1.25) SD units lower LDL cholesterol and −1.86 (95% CI, −2.56 to −1.16) lower VLDL cholesterol, when compared with heterozygous *ANGPTL3* LOF carriers and noncarriers. Many other lipids and lipoprotein measures also had substantially larger effect sizes for homozygous *ANGPTL3* LOF carriers in a recessive model (Figures IV and V in the online-only Data Supplement; Table III in the online-only Data Supplement). Of nonlipid markers, creatinine and acetate were significantly lower in *ANGPTL3* homozygotes.

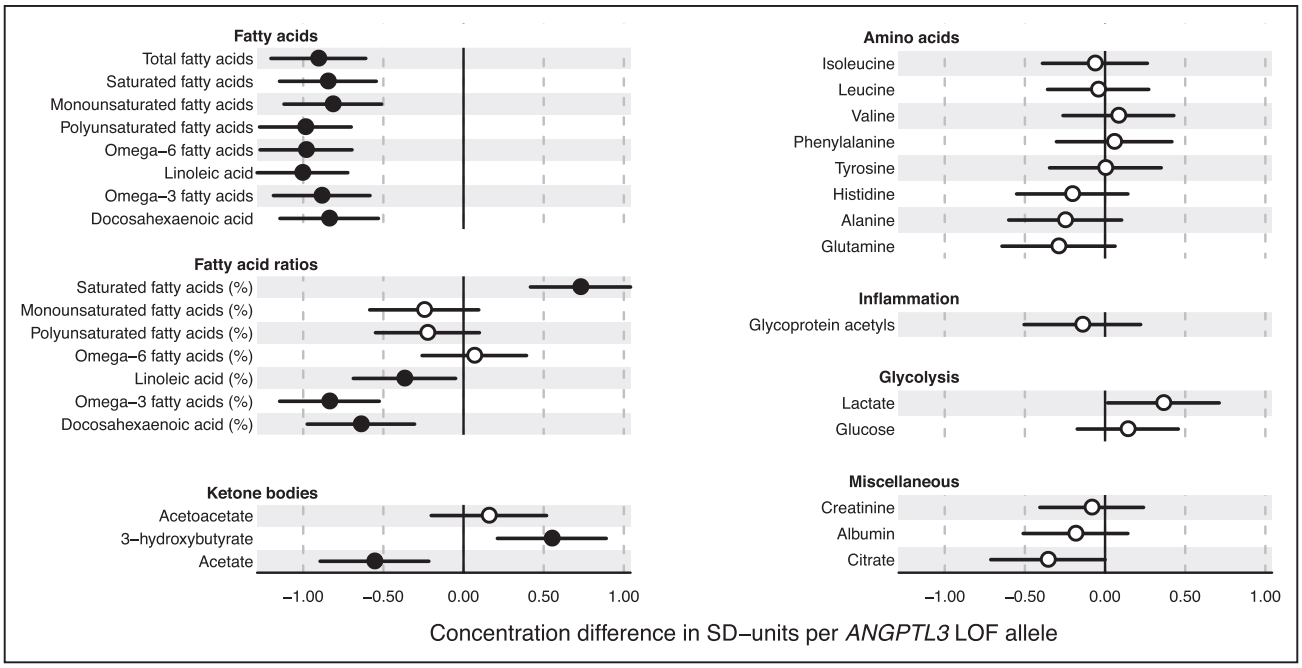

Figure 3. Effects of *ANGPTL3* (angiopoietin-like protein 3) loss-of-function (LOF) variant on fatty acids and polar metabolites. Effect estimates are shown as difference in SD-scaled concentration units per *ANGPTL3* S17X allele (additive model). Error bars indicate 95% CIs. Filled and open circles denote false discovery rate–corrected *P* value for association below and above 0.05, respectively.

Effects of *ANGPTL3* LOF on Metabolic Response to a Fat Challenge

To examine the effects of ANGPTL3 deficiency on postprandial metabolism, we further assessed the lipoprotein and metabolite trajectories after an oral fat challenge. Postprandial responses in selected metabolic measures for *ANGPTL3* LOF carriers and noncarriers are shown in Figure 4. *ANGPTL3* LOF homozygotes had drastically lower concentration of cholesterol in VLDL, LDL, and IDL particles in all timepoints in comparison with *ANGPTL3* LOF heterozygotes and noncarriers. There was essentially no change in VLDL cholesterol after the fat challenge among *ANGPTL3* LOF homozygotes, whereas *ANGPTL3* LOF heterozygotes and noncarriers showed substantially increased levels (P_{iAUC}=0.07 for difference in iAUC). This effect on VLDL cholesterol seemed to be driven particularly by differences for large and medium-sized VLDL particles in response to the high-fat meal. For instance, both *ANGPTL3* LOF heterozygotes and noncarriers displayed >80% increase in cholesterol in large VLDL at 4 hours after the meal when compared with *ANGPTL3* LOF homozygotes (P_{iAUC}=0.02 for *ANGPTL3* LOF homozygotes versus noncarriers). In contrast, the high-fat meal did not give rise to an increase in cholesterol levels of small VLDL, IDL, and LDL particles for any of the groups; rather, there was a small decrease in cholesterol within these smaller apoB-carrying lipoprotein particles.

The postprandial response to the oral fat challenge in total fatty acids, β-hydroxybutyrate, and lactate is shown in Figure 4. Homozygous *ANGPTL3* LOF carriers had markedly lower total fatty acids at all timepoints in comparison with the other 2 groups and showed essentially no postprandial increase in fatty acid concentrations. Although the absolute concentration of omega-3 fatty acids was essentially unaffected by the fat challenge, the proportion of omega-3 fatty acids to total fatty acids increased after the meal in *ANGPTL3* LOF homozygotes compared with other groups (P_{iAUC}=0.0003; Figure VI in the online-only Data Supplement). The responses in absolute levels of saturated and monounsaturated fatty acids followed similar patterns as that of total fatty acids, with essentially no postprandial response for *ANGPTL3* LOF homozygotes; however, the relative proportion of monounsaturated fatty acids increased for all groups (Figure VI in the online-only Data Supplement). The shape of the postprandial response in ketone bodies was broadly similar for *ANGPTL3* LOF carriers and noncarriers, but the concentrations of β-hydroxybutyrate were maintained elevated in the homozygous *ANGPTL3* LOF carriers (Figure 4). *ANGPTL3* LOF carriers also displayed consistently higher lactate levels after the fat meal challenge and increases in postprandial citrate and acetate levels. Among other nonlipid biomarkers, GlycA (a marker of chronic inflammation) levels showed a blunted postprandial response in *ANGPTL3* LOF homozygotes, possibly indicating a smaller inflammatory effect of the high-fat meal (P_{iAUC}=0.03; Figure 4). Postprandial responses for other selected biomarkers are shown in Figure VII in the online-only Data Supplement.

Discussion

In the present work, we describe a detailed metabolic signature associated with ANGPTL3 deficiency in humans. Because ANGPTL3 deficiency is known to influence postprandial lipid metabolism via modulation of lipoprotein lipase activity,[21] we considered it particularly interesting to investigate the fine-grained metabolic effects of *ANGPTL3* LOF also after a high-fat meal. We found that ANGPTL3 deficiency is characterized by markedly low levels of fasting and postprandial LDL and

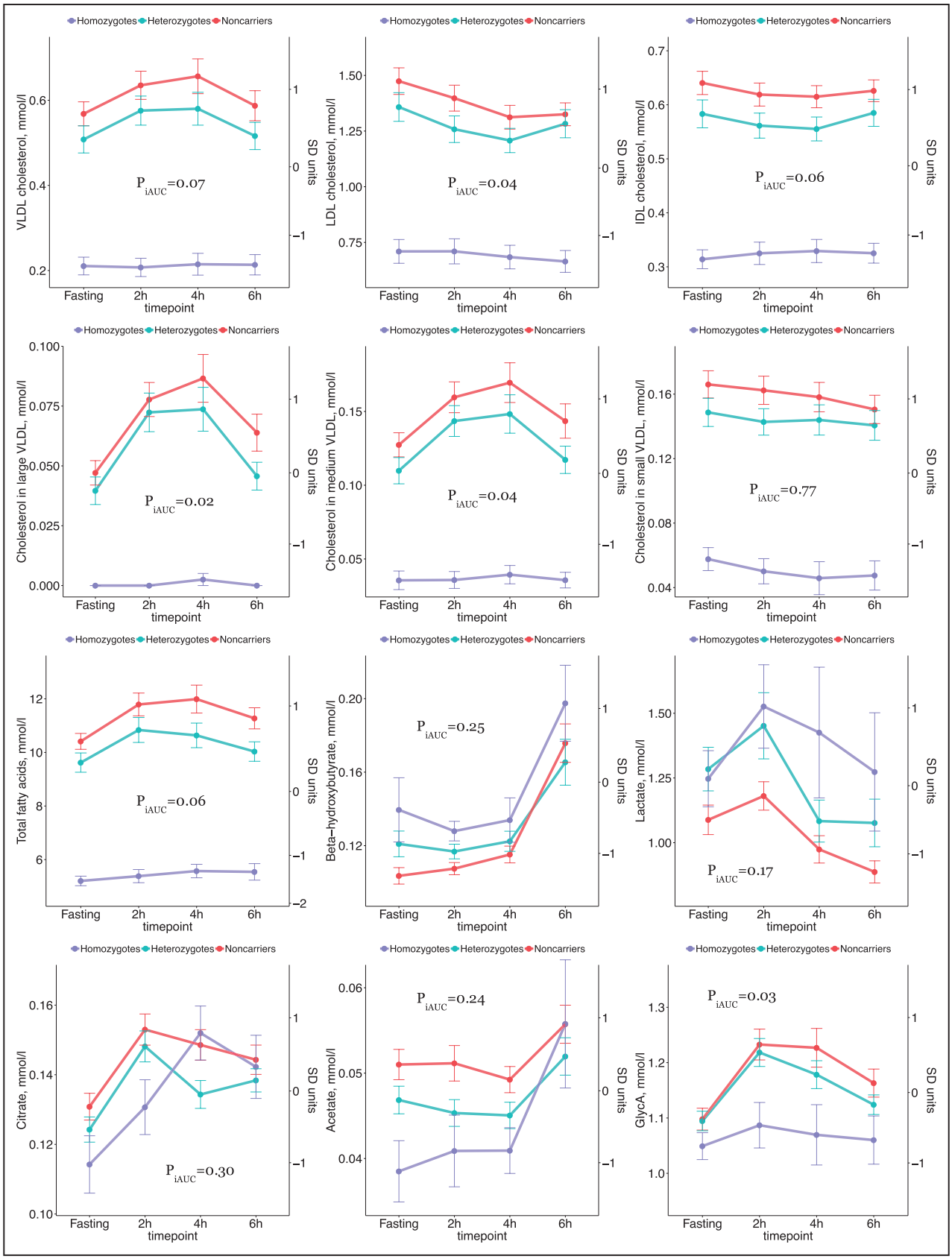

Figure 4. Effects of *ANGPTL3* (angiopoietin-like protein 3) loss-of-function (LOF) carrier status on metabolic response to an oral fat tolerance test. Results are shown as mean concentration and SEs at fasting and postprandial blood sample draw for selected biomarkers. Left axes indicate concentrations in absolute units, and right axes indicate concentrations in SD-scaled units. P_{iAUC} indicates significance of difference in incremental area under the curve, reflecting meal response for *ANGPTL3* S17X LOF homozygotes vs noncarriers. IDL indicates intermediate-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein.

VLDL cholesterol, as well as other measures of TRL. In particular, individuals with 2 inactivating mutations in *ANGPTL3* and no measurable concentration of ANGPTL3 protein showed essentially no increase in TRL in response to a high-fat meal. The low lipid levels among these homozygote carriers, who effectively act as human knockouts for *ANGPTL3*, were maintained after the oral fat challenge without evidence for adverse compensatory effects on the extensive metabolic biomarker panel examined in this study.

The main findings of our study are 4-fold: first, we found that ANGPTL3 deficiency results in similar reductions in magnitude for LDL and VLDL cholesterol, when scaled to the same variation in each lipid measure. Thus, *ANGPTL3* inhibitors should be more effective in lowering VLDL cholesterol as compared with statins or *PCSK9* inhibitors,[19,20] suggesting added benefit of *ANGPTL3* inactivating therapy in individuals with combined dyslipidemias. This finding has clinical relevance, because increasing evidence suggests that the cholesterol content of VLDL and other TRLs is causally implicated in the development of CVD, independent of LDL cholesterol.[2,3,27] Although TRLs have been suggested to play a causal role in CVD in Mendelian randomization studies,[28] the triglycerides per se are unlikely to be the causal factor because these lipids are not accumulating in the atherosclerotic plaque. In contrast, the cholesterol within TRLs and their remnants can accumulate in the arterial intima and even get trapped more easily than LDL cholesterol.[2] In line with our results, a recent phase 1 trial on antisense oligonucleotides targeting hepatic *ANGPTL3* mRNA has reported ≤60% reduction in VLDL cholesterol and 33% reduction in LDL cholesterol.[18]

Second, the detailed profiling of lipoprotein subclasses revealed that the absolute concentrations of both cholesterol and triglycerides in most lipoprotein subclasses were reduced in *ANGPTL3* LOF carries compared with noncarriers. However, the proportion of cholesterol (relative to the total lipid concentration in a given subclass) was reduced and the proportion of triglycerides increased in small and medium-sized VLDL and IDL subclasses. These results indicate that ANGPTL3 deficiency leads to less cholesterol load in the composition of TRL, which could potentially contribute to explain the lower CVD risk observed among *ANGPTL3* LOF carriers. We observed no clear differences in the patterns of postprandial response of TRL measures for *ANGPTL3* LOF heterozygotes as compared with noncarriers. In contrast, there was a remarkable lack of increase in TRL after the fatty meal intake among the 6 individuals with complete ANGPTL3 deficiency, as observed previously with narrower panel of lipid measures.[21,29] This lack of a dose-dependent postprandial response could suggest that therapeutic inhibition of *ANGPTL3* may only alleviate the increase in TRL after a meal intake if protein levels of ANGPTL3 are lowered to a high extent. To this regard, we have recently determined that plasma ANGPTL3 <60 ng/mL represents a threshold associated with marked reductions in fasting lipid and lipoprotein concentrations[30]; given the baseline plasma ANGPTL3 concentration among controls in the present study (Table), the lowering effects reported for ANGPTL3-LRx, ≥20 mg dose, should be sufficient to achieve ANGPTL3 levels below this threshold.[18] Further studies are required to elucidate whether this extent of plasma ANGPTL3 reduction is sufficient to substantially blunt the postprandial response in TRL or whether complete ANGPTL3 deficiency is required to observe this.

Third, we found that ANGPTL3 deficiency causes an elevation of fasting concentrations of the ketone β-hydroxybutyrate, which was maintained throughout the postprandial response. During ketogenesis in the liver, fatty acids are converted into ketone bodies via β-oxidation to produce energy. As ketogenesis is elevated when the influx of fatty acids into the liver is increased,[31] the present observation may indicate that complete ANGPTL3 deficiency is accompanied by an increased hepatic utilization of fatty acids derived from the lipolysis of TRL or from adipose tissue. However, because the shape of postprandial response in β-hydroxybutyrate and the other ketone body measured, acetoacetate, was similar independent of the *ANGPTL3* carrier status, one could speculate that this phenomenon might be related to partitioning of adipose tissue–derived fatty acid during the interprandial phase. However, this issue requires further investigations. Similarly to β-hydroxybutyrate, lactate levels were consistently higher at fasting and after the meal challenge for complete ANGPTL3 deficiency. This may indicate an enhanced conversion of pyruvate to lactate instead of its routing to the acetate pathway, consistent with the reduced concentration of acetate in the LOF carriers. These observations may suggest that ANGPTL3 deficiency is causing a modest shift of energy substrate utilization. The potential health effects of enhanced ketone body production remain unclear, but large epidemiological studies have generally reported only weak or null associations of ketone bodies with cardiometabolic risk factors and disease outcomes.[22]

A further insight from this study arises from the metabolic biomarkers not associated with ANGPTL3 deficiency, in particular for the homozygous carriers. These null results suggest there are no substantial adverse effects of complete absence of ANGPTL3 protein on amino acids and glycolysis-related metabolites, neither at fasting nor as a compensatory mechanism for the absence of postprandial lipid increase. This is informative for the safety of *ANGPTL3*-inhibiting therapeutics, because many of these metabolites have been shown to be biomarkers of the risk for type 2 diabetes mellitus and CVD events.[23,26] This is also the case for several of the relative proportions of fatty acid analyzed in this study. The observed modulations in the fatty acid balance for the relative proportions of saturated and omega-3 fatty acids could potentially be unfavorable in terms of cardiovascular risk, but the causal relations of these measures remain unclear. For the postprandial effects, it is noteworthy that the inflammatory biomarker GlycA was low in *ANGPTL3* LOF homozygotes and less elevated in the postprandial stage; such blunted increase in low-grade inflammation in response to a fatty meal might contribute to additional risk-reducing effects beyond lipids. Overall, these results illustrate the potential of wider metabolic profiling of human knockouts for drug targets for elucidating molecular mechanisms and potential metabolic side effects.

The strengths of the present study include a unique setting with fasting and postprandial measurements in *ANGPTL3* LOF carriers, including the rare instance of 6 individuals with complete ANGPTL3 deficiency. We used

NMR metabolomics to obtain detailed measures of lipid metabolism and nonlipid biomarkers from multiple metabolic pathways. There are also potential limitations in our study. First, we acknowledge that other metabolomics assays capable of capturing additional blood metabolites could further contribute to characterize the metabolic effects beyond lipid metabolism. Second, despite the large effects on many lipid levels caused by ANGPTL3 deficiency, the small sample size provided limited power to detect all relevant metabolic associations, in particular for robustly comparing differences in the postprandial response. Third, the biophysical properties of triglyceride-rich chylomicrons and the largest VLDL particles that are elevated in the postprandial state may impair the accuracy of the NMR-based quantification of lipids in these particles. This can lead to an underestimation of the postprandial lipid concentration in these large lipoprotein particles, as observed previously.[24] In the present study, this may cause a slight underestimation of the postload differences of VLDL lipids between individuals with complete ANGPTL3 deficiency compared with heterozygotes and noncarriers. As illustrated in Figure I in the online-only Data Supplement, the spectral signals originating from lipoprotein lipids are almost exclusively confined to 2 large peaks, and the effects of chylomicrons do generally not affect the quantification of other metabolites in different spectral areas or to the accuracy of smaller lipoprotein particles. Finally, our study was limited to characterize carriers of *ANGPTL3* S17X LOF mutation only. Thus, there is a need for further metabolic characterization of other LOF variants in the *ANGPTL3* gene. Detailed metabolic profiling in large biobanks with sequencing information available provides an opportunity to enable this and further extend applications to other lipid-lowering targets.[20,32]

In conclusion, this detailed metabolic profiling study demonstrates that ANGPTL3 deficiency is characterized by substantial reductions in VLDL particles and their remnants and a lower cholesterol content in these lipoproteins. Further, complete ANGPTL3 deficiency leads to virtual absence of postprandial increase in TRL. These findings support the increasing body of evidence indicating that genetic inhibition of *ANGPTL3* causes a broad range of beneficial lipid changes, without adverse compensatory metabolic effects. Detailed metabolic profiling in trials of pharmacological inactivation of *ANGPTL3* could further help to confirm these findings in clinical settings and, in combination with the genetic evidence, uncover potential off-target effects.[33]

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Highlights

- ANGPTL3 (angiopoietin-like protein 3) deficiency results in similar reductions in LDL (low-density lipoprotein) cholesterol and many triglyceride-rich lipoprotein lipids measures, such as VLDL (very-low-density lipoprotein) cholesterol, with no evidence of substantial adverse effects on the comprehensive panel of circulating metabolite biomarkers tested here.
- In particular, ANGPTL3 deficiency results in reduction of cholesterol content in triglyceride-rich lipoproteins and their remnants, which have been highlighted as risk factor for cardiovascular disease independently of LDL levels.
- Homozygous *ANGPTL3* loss-of-function carriers show essentially no postprandial increase in triglyceride-rich lipoproteins and fatty acids in response to a fat challenge and display consistently elevated postprandial levels of ketone body β-hydroxybutyrate when compared with non-carriers, suggesting enhanced hepatic fatty acid β-oxidation.